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Soil microbial activity and community structure as affected by exposure to chloride and chloride-sulfate salts

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Abstract: Mixed or chloride salty ions dominate in saline soils, and exert wide-ranging adversely affect on soil biological processes and soil functions. The objectives of this study were to (1) explore the impacts of mixed (0, 3, 6, 10, 20 and 40 g Cl^-/SO_4^{2-} salt/kg dry soil) and chloride (0, 1.5, 3, 5, 8 and 15 g Cl^- salt/kg dry soil) salts on soil enzyme activities, soil physiological functional (Biolog) profiles and microbial community structure by using soil enzymatic, Biolog-Eco microplates as well as denaturing gradient gel electrophoresis (DEEG) methods, and (2) determine the threshold concentration of soil electronic conductivity ($EC_{1:5}$) on maintaining the functional and structural diversity of soil microbial community. The addition of either Cl^- or mixed Cl^-/SO_4^{2-} salt obviously increased soil EC, but adversely affected soil biological activities including soil invertase activity, soil microbial biomass carbon (MBC) and substrate-induced respiration (SIR). Cl^- salt showed a greater deleterious influence than mixed Cl^-/SO_4^{2-} salt on soil enzymes and MBC, e.g., the higher soil MBC consistently appeared with Cl^-/SO_4^{2-} instead of Cl^- treated soil. Meanwhile, we found that SIR was more reliable than soil basal respiration (SBR) on explaining the changes of soil biological activity responsive to salt disturbance. In addition, microbial community structures of the soil bacteria, fungi, and *Bacillus* were obviously affected by both salt types and soil EC levels, and its diversity increased with increasing of mixed Cl^-/SO_4^{2-} salt rates, and then sharply declined down after it reached critical point. Moreover, the diversity of fungal community was more sensitive to the mixed salt addition than other groups. The response of soil physiological profiles (Biolog) followed a dose-response pattern with Cl^- (R^2=0.83) or mixed Cl^-/SO_4^{2-} (R^2=0.89) salt. The critical threshold concentrations of salts for soil physiological function were 0.45 dS/m for Cl^- and 1.26 dS/m for Cl^-/SO_4^{2-}, and those for soil microbial community structural diversity were 0.70 dS/m for Cl^- and 1.75 dS/m for Cl^-/SO_4^{2-}.

Keywords: soil biological activity; microbial diversity; chloride salt; mixed salt; threshold concentration

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1 Introduction

Soil salinity is a significant constraint on agricultural production. It was reported that over 6% of the world's land is affected by soil salinity, accounting for >800×10^6 hm^2 of land (Munns and Tester, 2008). In arid and semi-arid regions, soil salinity has already reduced the potential crop production by ca. 20% (Porcel et al., 2012). In China, the total area of saline soil is about 36×10^6 hm^2, accounting for 4.88% of the country's total available land area (Wang et al., 2011). As soil salinization usually triggers by tillage practices, drainage and irrigation, intensive agricultural land is particularly at risk of degradation caused by salinization. Globally, ca. 0.5%–1.0% of irrigated areas is abandoned annually due to the deleterious impacts of salinity (FAO, 2002), and the annual cost caused by soil salinization is estimated to be greater than US$ 27.3×10^9 (Qadir et al., 2014).

Salinity impacts agricultural production through deteriorations of soil physical-chemical properties such as permeability, aggregate stability, and soil structure (Morrissey et al., 2014; Qadir et al., 2014), which in turn have concomitant impacts on soil microbial community diversity and function (Rietz and Haynes, 2003; Cortés-Lorenzo et al., 2014), and these underpin provisioning services (production of food and fiber), regulating services (nutrient cycling, soil C storage, etc.) and supporting natural capital (the extent of biological diversity in soil) (Dominati et al., 2010).

García and Hernández (1996) pointed out that high soil electronic conductivity (EC), a result of accumulation of mobile salts in soils, resulted in the reduction of soil aggregate stability and the decrease of soil biological activity. Following this, Wakelin et al. (2012) and Morrissey et al. (2014) found the obvious shifts in bacterial abundance and community composition along with soil EC gradients. However, different types and levels of soil salt exhibit different impacts on soil biological property and function. For example, soil nitrification rate is increasingly inhibited by the increase of soil EC (Inubushi et al., 1999). Rath et al. (2016) showed that microbial respiration was more strongly affected by Cl^- than by SO_4^{2-} containing salts. Nevertheless, controversial results often exist in literature about the relative impact of salt type and concentration on soil biological property (Rath and Rousk, 2015; Rath et al., 2016). Considering the increasing concerns of the soil salinization problem, further understanding the impacts of salt on soil biological process is urgently needed.

In the present study, the impacts of chloride (Cl^-) and mixed salt (Cl^-/SO_4^{2-}) gradients on soil microbial communities and enzyme activities were assessed. The objectives of this study were to (1) explore the influences of Cl^- and mixed Cl^-/SO_4^{2-} salts on soil biological characteristics including soil enzyme activity, physiological function, and soil microbial community composition and diversity; and (2) determine the threshold concentration of salinity ($EC_{1:5}$) in maintaining soil microbial community function and structural diversity.

2 Materials and methods

2.1 Site description and soil sampling

The soil samples were collected from the Wulanwusu Meteorological Station (44°17′N, 85°49′E), located in Shawan County, Xinjiang, China. The site has an annual average temperature of 7.0°C, an average annual precipitation of 211 mm, and an average annual evaporation of 1664 mm. The soil is classified as a cultivated grey desert soil (Calcaric Fluvisol, World Reference Base for Soil Resources), with the following physical-chemical properties: soil $EC_{1:5}$ of 0.16 dS/m, organic content of 5.03 g/kg, and field moisture capacity of 24.9%.

Soil cores (0–20 cm in depth and 5 cm in diameter) were collected at random after removing the litter, and were thoroughly mixed to form a representative composite sample of this area. Plant residues and visible animals (e.g., invertebrates) were manually removed, then soil sample was sieved (<2 mm) and stored at 4°C. Before the salt-dose incubation experiment, pre-experiment was conducted to activate soil microbial activity at the 50% of the water holding capacity for 7 d.

2.2 Mesocosm experiment

A mesocosm incubation experiment was set up to investigate the impacts of salt type and rate on soil biological properties. Chloride saline gradient (Cl^- treatments, i.e., 1.5, 3, 5, 8, and 15 g Cl^-/kg dry soil) was simulated by combining $CaCl_2$ and NaCl with the molar ratio of 1:1, while mixed saline gradient (mixed Cl^-/SO_4^{2-} treatments, i.e., 3, 6, 10, 20, and 40 g Cl^-/SO_4^{2-}/kg dry soil) was simulated by combining $CaCl_2$ and Na_2SO_4 with the molar ratio of Cl^- and SO_4^{2-} of 3:1. All these two-salt gradients were sterilized using autoclave (121°C, 100 kPa for 30 min) before mixed them with soil. For both salt treatments, five increasing levels of salt were thoroughly mixed into 1 kg samples of sieved soil. The soil receiving neither Cl^- nor Cl^-/SO_4^{2-} was regarded as the control treatment (CK).

An aliquot of 1 kg soil was taken in each plastic pot (13 cm height×11 cm bottom diameter), and each treatment had three replicates. All treatments incubated 31 d in a growth chamber at a constant temperature of 25 (±2)°C in the dark condition. The sterilized Milli-Q water was added daily to maintain soil moisture at the water holding capacity of 60% during the whole incubation period. The level of soil salinity in this study was expressed using soil EC (dS/m, 1:5, soil:water), and was measured by a DDS-307 conductivity meter (INESA, Shanghai, China; Rath and Rousk, 2015).

At the end of the incubation, each soil sample was thoroughly mixed and divided into three portions. One was air-dried to determine soil EC. The second portion was stored at 4°C prior to analysis of microbial biomass carbon (MBC), soil basal respiration (SBR), enzyme activity (within 3 to 7 d) and soil physiological profiles. The third portion was stored at –80°C before used for DNA-based analysis of the microbial community composition. All soil microbial parameters were measured three times (total soil sample for each parameter=treatments×replicates). Soil moisture determination paralleled with the measurements of soil microbial culture-independent parameters and soil enzyme activities. All soil parameters are presented as soil dry weight (105°C) basis.

2.3 Soil enzyme activities

According to the methods for soil enzyme activity analysis (Guan et al., 1986), we measured catalase activity using the potassium permanganate titration method. Briefly, 1 g of air-dried soil was titrated with 0.1 mol/L $KMnO_4$, and the volume of each titration was measured and the activity was calculated. Similarly, we estimated soil polyphenol oxidase activity by the pyrogallol method based on the content of gallic acid that was generated during the process. Soil invertase activity was measured by the 3, 5-dinitrosalicylic acid colorimetric method at 508 nm.

2.4 MBC and SBR

We determined MBC (an indicator of the overall size of the soil microbe) using the fumigation–extraction method according to Vance et al. (1987). In brief, fumigated and non-fumigated soils (10 g dry weight) were extracted with 40 mL of 0.5 M K_2SO_4 (soil/extractant ratio 1:4); all soil samples were shaken for 30 min, filtered through a 0.45-μm glass fiber filter, and then used for measurement of C on a total organic carbon (TOC) analyzer (TOC-5000A, Shimadzu, Kyoto, Japan). The amount of MBC was calculated by Equation 1.

$$MBC=(\text{total C amount in fumigated extract}-\text{total C amount in non-fumigated extract})\times 0.38, \quad (1)$$

where 0.38 is a conversion factor for C (Vance et al., 1987).

SBR (an indicator of the overall microbial activity) was measured by placing 30 g air-dry soil into 50 mL beakers and the sample was then incubated at 25°C in the dark in a 1-L air-tight sealed jar along with 10 mL of 1 M NaOH. The CO_2-C evolved was determined by titration after 2, 5, and 10 d, respectively (Anderson, 1982).

2.5 Substrate induced respiration and community-level physiological profiling

Soil microbial physiological functional profile was measured by Biolog-Eco microplates (Biolog Co., Hayward, CA, USA). Each Eco-plate contained 3 replicate sets of 31 ecologically-relevant C substrates along with a no-C treatment (control). Briefly, 2.6 (±0.1) g soil was weighed into a

50-mL centrifuge tube and 27 mL of phosphate-buffered saline (PBS; pH 6.8) added. The samples were shaken by end-over-end tumbling for 2 h to completely disperse soil particles. A second 1:10 dilution was made into PBS and mixed for a further 30 min. Aliquots were then loaded into Biolog-Eco microplates (100 mL per well).

Color development in each well was determined at the start of the experiment (T_0) using a Biolog Micro station™ (BIO-TEK Instruments Inc., Winooski, VT, USA) at 590 nm, and then at 12 h intervals over a 120-h incubation period (28°C, dark). At each sampling time, the color development of each substrate was calculated within each plate.

For Biolog analysis, OD_{590} value of the control (water) was subtracted from the value of each substrate-containing well. The average well color development (AWCD), representing multiple SIR to 31 C sources, was calculated for each sampling time over the 120-h incubation (Wekelin et al., 2010). For each sample, the AWCD was integrated over time (the area under the curve; AUC) to produce an AWCD-AUC statistic representing multiple SIR for each sample.

2.6 DNA extraction and biological community structure and diversity

Soil DNA was extracted from 0.7 g of soil using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories Inc., USA) following the manufacturer's instruction. DNA extracts were eluted into TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and stocks stored at –80°C. Working solutions of 1/10 dilution of stock DNA in sterile, DNA-free water were used for PCR. These solutions were stored at –20°C.

Soil *Bacillus* can produce a wealth of antibacterial substances including lipopeptides, polypeptides, and phospholipids, reflecting the capacity of abiotic stress tolerance (Han et al., 2014). Thus, soil bacterial, fungal, and *Bacillus*-specific PCRs were conducted on the soil DNA samples. The PCR methods for these have been described fully by Heuer and Smalla (1997), Garbeva et al. (2003) and Wakelin et al. (2007).

Community profiles were based on denaturing gradient gel electrophoresis (DGGE) fingerprint profiles analysis. DGGE was performed using a DCode System (BioRad, Laboratories, Hercules, CA, USA). Profiling of total bacteria used a 7% acrylamide: bis-acrylamide (37.5:1.0) gel with a formamide and urea denaturing range of 45%–55% (Wakelin et al., 2012). For soil fungi, a 6% polyacrylamide gel with a 30%–50% denaturing range was used (Wakelin et al., 2007). For *Bacillus*, DGGE was conducted using 6% acrylamide gels with a 35%–75% denaturing range (Wakelin et al., 2010). For all DGGE, separation was generated using 1×TAE buffer at 60°C, with 90 V, and for 17 h (Wakelin et al., 2010).

DGGE gels were stained using SYBR gold (1×TAE buffer; molecular probes) for 30 min and visualized on a DarkReader (Clare Chemicals Inc., USA). Gel images were digitally captured using an Olympus E-500 digital SLR camera and the position and intensity of bands were determined using Gel-Quant software. The Shannon index (H'; Eq. 2) was calculated from the profiles of bacteria, fungi, and *Bacillus* communities.

$$H' = -\sum p_i \times \ln p_i, \tag{2}$$

where p_i is the proportion of members that a particular species i contributes to the total in the sample (Shannon and Weaver, 1949).

2.6.1 PCR of bacteria

The primer universal pair F968-GC-R1404 (Heuer and Smalla, 1997) was used to amplify soil bacterial 16S rRNA genes (Table 1). Each 25 μL reaction contained 4 μL template DNA, 1 μL of each 10 mM primer, 10 μL of 2×Taq mix (Cwbiotech, China), and 9 μL of DNA-free ddH_2O. The PCR cycling was: denaturation at 94°C for 5 min, followed by 30 cycles of denaturing for 1 min at 94°C, annealing for 1 min at 67°C and extension for 1 min at 72°C (annealing temperature lowered 0.5°C per cycles until reaching 57°C), then another 18 cycles of denaturing for 1 min at 94°C, annealing for 1 min at 57°C, and extension for 1 min at 72°C. The PCR was followed by a final extension for 20 min at 72°C. The product of fragment length is 450 bp.

2.6.2 PCR of fungi

The primer universal pair ITS1F-GC-ITS2 (Wakelin et al., 2007) was used to amplify soil fungi ITS rRNA genes (Table 1). Each 25 μL reaction contained 6 μL template DNA, 0.8 μL of each 10

mM primer, 10 μL Taq mix (Cwbiotech, China), and ddH_2O to reach 25 μL. The PCR program was as follows: denaturing step of 94°C for 5 min, followed by 36 cycles of denaturing for 1 min at 94°C, annealing for 30 s at 58°C, and extension for 1 min at 72°C, and a final extension for 10 min at 72°C. The product of fragment length is 300 bp.

2.6.3 PCR of *Bacillus*

Bacillus, group-selective community profiling was based on the nested PCR method of Garbeva et al. (2003). Primers BACF and R1378 were used in the first-round PCR. Primers BACF and R1378 were used in the first-round PCR at 0.2 mM each (Table 1). The reaction mixture also contained 1U of HotStar Taq polymerase (Qiagen), 2.5 μL of 10×buffer, 10 mM of each dNTP, 2 mL of 1/10 diluted DNA, and ddH_2O to a final volume of 25 μL. First round PCR used a touchdown thermos-cycling profile. Following hot-start polymerase activation (5 min at 94°C), denaturation was conducted at 95°C for 1 min, extension at 68°C for 2 min. Annealing was initially at 63°C (1 min) and reduced by 2°C per cycle until reaching 55°C. A further 21 cycles with a 55°C annealing temperature completed the PCR. Second-round PCR was used primers F968-GC and R1378 with 1:100 diluted of the first-round PCR product as template. Otherwise, the chemistry and PCR conditions were as for general bacteria.

Table 1 Primers for the amplifications of soil bacteria, fungi, and *Bacillus*

Treatment	Amplification reaction	Primer	Sequence (5'–3')	Product size (bp)	Reference
Bacteria		F968-GC	5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3'	450	Heuer and Smalla (1997)
		R1401	5'-GCG TGT GTA CAA GAC CC-3'		
Fungi		ITS1F-GC	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CTT GGT CAT TTA GAG GAA GTA A-3'	300	Wakelin et al. (2007)
		ITS2	5' -TTY GCT GYG TTC TTC ATC G-3'		
Bacillus	1st round	BACF	5'- GGG AAA CCG GGG CTA ATA CCG GAT-3'	1300	Garbeva et al. (2003)
		R1378	5'- CGG TGT GTA CAA GGC CCG GGA ACG-3'		
	2nd round	F968-GC	5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAG AAC CTT AC-3'		
		R1378	5'-CGG TGT GTA CAA GGC CCG GGA ACG-3'		

2.7 Statistical analyses

The relationships between soil enzyme activity, MBC, SBR, and SIR with salt induced soil EC were analyzed using linear regression. Regressions in slope between the Cl^- or mixed Cl^-/SO_4^{2-} with soil salt levels were tested to determine salt-specific effects. All above analyses were conducted in Prism7 (GraphPad Software, San Diego, CA, USA).

The influences of salt type and rate-affected on physiological profiles (microbial mineralization of 31 carbon sources) and community composition (the assemblages of bacteria, fungi, and *Bacillus* communities) were tested using a multivariate approach. The physiological profiles (C-utilization plates; Biolog Inc., Hayward, CA, USA) were analyzed after 120 h incubation. This time point was chosen because it allows suitable time for color development in the wells with high salinity, while the color development for the control samples was still linearly increasing over time. The responses of the variables were normalized and the similarity in C-use among samples was compared using the Euclidean distance method. We tested the effects of salt type (Cl^- and mixed Cl^-/SO_4^{2-}) and salt-induced changes in soil EC using permutation-based multivariate analysis of variation (PERMANOVA; Anderson, 2001), with salt type as the main test, and EC as a covariate. For the bacterial and fungal community analysis, PCR-DGGE band intensity data were 4^{th}-root transformed (down-weighting over abundant species) and similarities in community compositions among the samples were calculated using the Bray-Curtis algorithm. The inverse Simpson index (1-λ, soil diversity) was calculated with non-transformed DGGE band

data (Primer 6). Multivariate statistical testing then followed the PERMANOVA approach (as above). Segmental dose-response logistic regressions were fitted to model the effects of salt treated gradient on AWCD (120 h). And we calculated the IC50 values (concentration of a contaminant that inhibits 50% of a specific biological process, referred as threshold) using the least-squares method (GraphPad Prism version 5, GraphPad Software, San Diego; Haanstra et al., 1985). Based on 16S-rDNA and ITS-rDNA gene PCR-DGGE banding profiles, we calculated the relationship between soil diversity and salt treated gradient using segmental linear regression and the fit of the data compared with single linear regression alone. The intercepts of segmental regressions were used to determine threshold effects of increasing salt on soil microbial community diversity.

3 Results

3.1 Soil EC

The influences of both Cl^- and mixed Cl^-/SO_4^{2-} salts on soil EC are given in Table 2. The average EC was 0.16 dS/m in the control. This falls well under the standard threshold for saline soils (EC of the saturation soil extract >4 dS/m at 25°C). The addition of either Cl^- or mixed Cl^-/SO_4^{2-} salt highly increased soil EC.

Table 2 Dosage of Cl^- and mixed Cl^-/SO_4^{2-} added to soil, and the corresponding soil electrical conductivity ($EC_{1:5}$)

Salinity type	Salinity gradient	Soil $EC_{1:5}$ (dS/m)			Average $EC_{1:5}$[c]
		Replicate 1	Replicate 2	Replicate 3	
Control	CK	0.14	0.19	0.15	0.16±0.03
Cl^- saline[a]	1.5 Cl^-	0.46	0.41	0.65	0.51±0.13
	3 Cl^-	1.09	1.04	0.81	0.98±0.15
	5 Cl^-	1.56	1.53	1.52	1.54±0.02
	8 Cl^-	1.92	1.69	1.81	1.81±0.16
	15 Cl^-	3.49	3.43	3.33	3.42±0.08
Cl^-/SO_4^{2-} saline[b]	3 Cl^-/SO_4^{2-}	0.90	0.79	0.80	0.83±0.06
	6 Cl^-/SO_4^{2-}	1.47	1.49	1.47	1.48±0.01
	10 Cl^-/SO_4^{2-}	2.26	2.21	2.28	2.25±0.04
	20 Cl^-/SO_4^{2-}	3.96	3.74	3.75	3.82±0.12
	40 Cl^-/SO_4^{2-}	6.13	6.17	6.04	6.11±0.07

Note: [a], chloride saline (Cl^- saline) was a mixture of $CaCl_2$+NaCl. [b], mixed saline (Cl^-/SO_4^{2-} saline) was a mixture of $CaCl_2$ and Na_2SO_4. [c], values represent mean±SD (n=3).

3.2 Soil enzyme activities

Both Cl^- and mixed Cl^-/SO_4^{2-} added treatments significantly and adversely affected invertase activity, yet had little influence on catalase or polyphenol oxidase (Figs. 1a and b). We compared the relative influences of Cl^- and mixed Cl^-/SO_4^{2-} salts on invertase activity based on the reduction in enzymatic activity against soil EC (Fig. 1c). The linear regression fit for the Cl^- data was statistically significant (R^2=0.964, P<0.001), and it was strong for the mixed Cl^-/SO_4^{2-} salt data (R^2=0.674, P=0.045). However, when the slopes of the two regression lines were compared, it was found that the reduction in invertase activity in Cl^- treated soils was greater than that in mixed Cl^-/SO_4^{2-} salt treatment, implying Cl^- salt was more toxic than mixed Cl^-/SO_4^{2-} salt on soil enzyme activity.

3.3 Microbial biomass, basal respiration, and substrate induced respiration

Salt addition significantly decreased soil MBC (Fig. 2a). When soil receiving the mixed Cl^-/SO_4^{2-} salt, the reduction was sharp and linear across the EC range (R^2=0.876, P=0.006). However, the relationship between MBC and EC was weaker (R^2=0.60, P=0.700) as soil receiving Cl^- salt. The

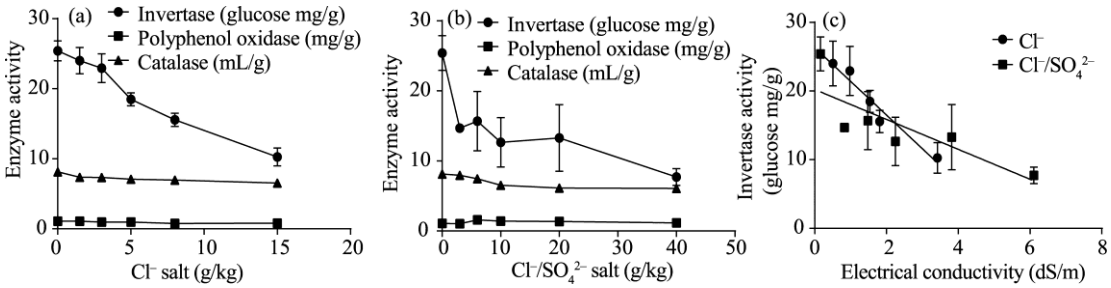

Fig. 1 Effects of Cl^- (a) and mixed Cl^-/SO_4^{2-} (b) salts on soil invertase, polyphenol oxidsase and catalase activities. Relationship between invertase activity and electrical conductivity for mixed Cl^-/SO_4^{2-} and for Cl^- treated soils (c). Error bars are standard deviations.

consistently greater soil MBC was gained with mixed Cl^-/SO_4^{2-} salt than with Cl^- salt treatment, indicating that Cl^- had greater detrimental impact than mixed Cl^-/SO_4^{2-} salt on MBC at equivalent salt rate. SBR was faintly affected by both Cl^- (P=0.289) and mixed Cl^-/SO_4^{2-} (P=0.312) added treatments (Fig. 2b). However, SIR (AWCD generated, 120 h AUC data) was sensitively responsive to the saline-induced changes in EC (Figs. 2b and c, and Fig. 3). The relationship between SIR in Cl^- treated soil and soil EC was not linear (P=0.339), while the relationship was strong (P=0.063) with the mixed Cl^-/SO_4^{2-} salt treated soil, indicating that SIR was more seriously affected by salt concentration than by salt type.

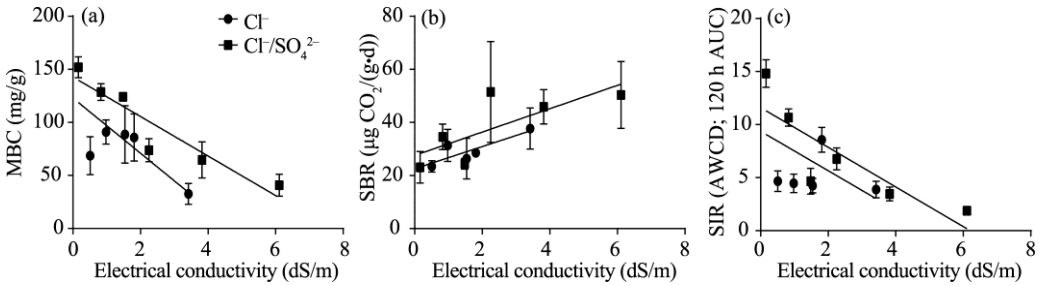

Fig. 2 Relationships between microbial biomass carbon (MBC, a), soil basal respiration (SBR, b) and substrate-induced respiration (SIR, c; average well color development (AWCD) generated, 120 h AUC data) with EC caused by Cl^-/SO_4^{2-} or Cl^- addition. Error bars are standard deviations.

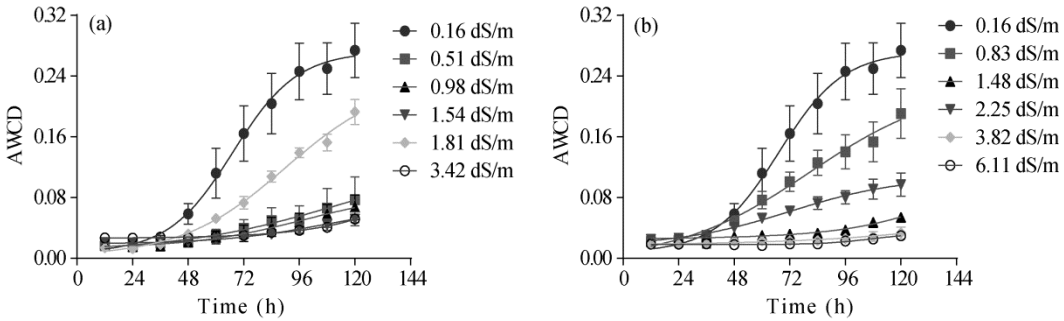

Fig. 3 Average well color development (AWCD) as affected by Cl^- (a) and mixed Cl^-/SO_4^{2-} salts (b). Error bars are standard deviation, n=18.

3.4 Physiological profiles, soil microbial community composition, and diversity

The dissimilarities of carbon metabolic profiles (Figs. 4a and b) and community structures of bacteria, fungi and *Bacillus* (Figs. 4c–h) with Cl^- or mixed Cl^-/SO_4^{2-} addition were shown in nMDS (non-metric multidimensional scaling ordinations) plots. Salt type had no obvious influence on soil physiological profiles (Table 3), however, salt levels (alteration of soil $EC_{1:5}$) highly impacted on soil physiological profiles (P=0.004). The strong shifts in soil physiological profile occurred with both Cl^- and mixed Cl^-/SO_4^{2-} salts (Figs. 4a and b). In terms of soil

community-level physiological profiles (AWCD value), the threshold values were 1.26 dS/m for mixed Cl^-/SO_4^{2-} salt and 0.45 dS/m for Cl^- salt indicating that the threshold concentrations of EC were 1.26 and 0.45 dS/m for mixed Cl^-/SO_4^{2-} and Cl^- salts.

Furthermore, structures of soil bacterial, fungal and *Bacillus* communities were obviously affected by both salt type and final soil EC (Figs. 4c–h; Table 3). For soil bacterial community structure, the proportions of variation explained by salt type (√CV=9.18) and EC (√CV=7.48) were similar, indicating both treatments exerted identical effects. A similar influence of the soil salt type and final soil EC on soil fungal community composition was observed (Table 4). For the composition of the soil *Bacillus* community (Table 3), only less variation was explained by salt type (√CV=8.73) than EC (√CV=5.73), indicating the effect of salt type on soil *Bacillus* was greater than soil EC.

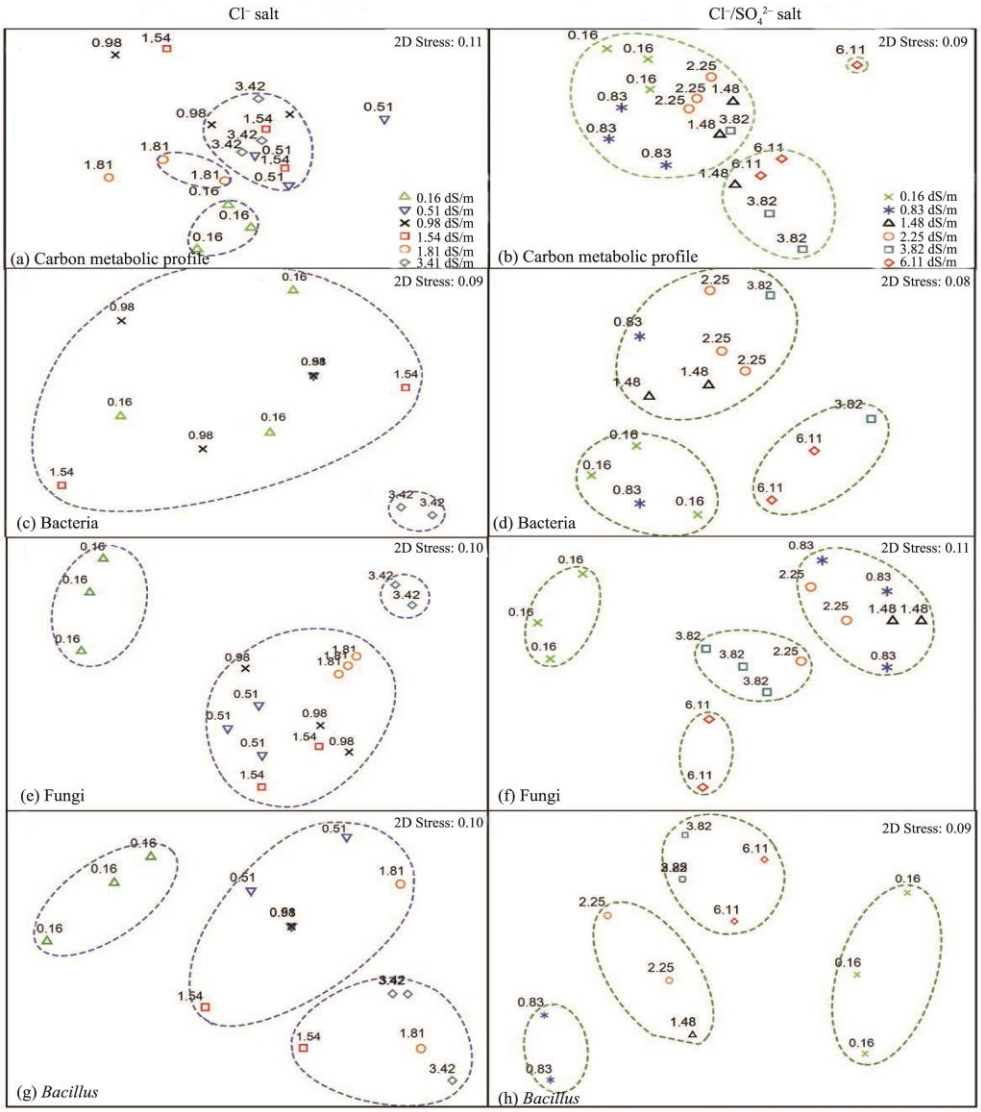

Fig. 4 nMDS (non-metric multidimensional scaling ordinations) plots show the dissimilarities in microbial catabolic functional profiles (Biolog-Eco mircroplates, 120 h) with Cl^- salt (a) and mixed Cl^-/SO_4^{2-} salt (b); soil bacterial community structures with Cl^- salt (c) and mixed Cl^-/SO_4^{2-} salt (d); soil fungal community with Cl^- salt (e) and mixed Cl^-/SO_4^{2-} salt (f); soil *Bacillus* community with Cl^- salt (g) and mixed Cl^-/SO_4^{2-} salt (h). Increasing distance between sample points reflects the increase of dissimilarity in overall functional profile or community structure. Samples cycled by ovals are significantly similar at P=0.05. The low stress values (all<0.11) indicate accurate two-dimensional scaling (representation) of the ecological distances within the multivariate data sets and each point is a sample.

Table 3 Permutation-based multivariate analysis of variation

Item	Physiological profiles		Fungal community		Bacterial community		*Bacillus* community	
	P (perm)	√CV	P (perm)	√CV	P (perm)	√CV	P (perm)	√CV
EC	0.004	1.50	0.012	4.78	0.001	7.48	0.001	5.73
Salt type	0.348	0.40	0.077	4.58	0.004	9.18	0.001	8.73
EC×salt	0.037	1.46	0.002	10.78	0.289	2.61	0.005	7.66
Residual		5.29		16.39		12.81		11.72

Note: P (perm) was calculated on the basis of 999 random permutations of the data set. √CV is the square root of the components of variance.

The diversities of bacterial, fungal and *Bacillus* communities initially increased with the increase in the mixed Cl^-/SO_4^{2-} salt till reaching a peak point, and then sharply declined (Fig. 5). Moreover, the diversity threshold of soil fungal community with mixed Cl^-/SO_4^{2-} added soil (EC=1.75 dS/m) was lower than that of *Bacillus* (EC=1.96 dS/m) and bacteria (EC=3.03 dS/m) (Table 4), suggesting that soil fungal community was more susceptible than soil bacteria or

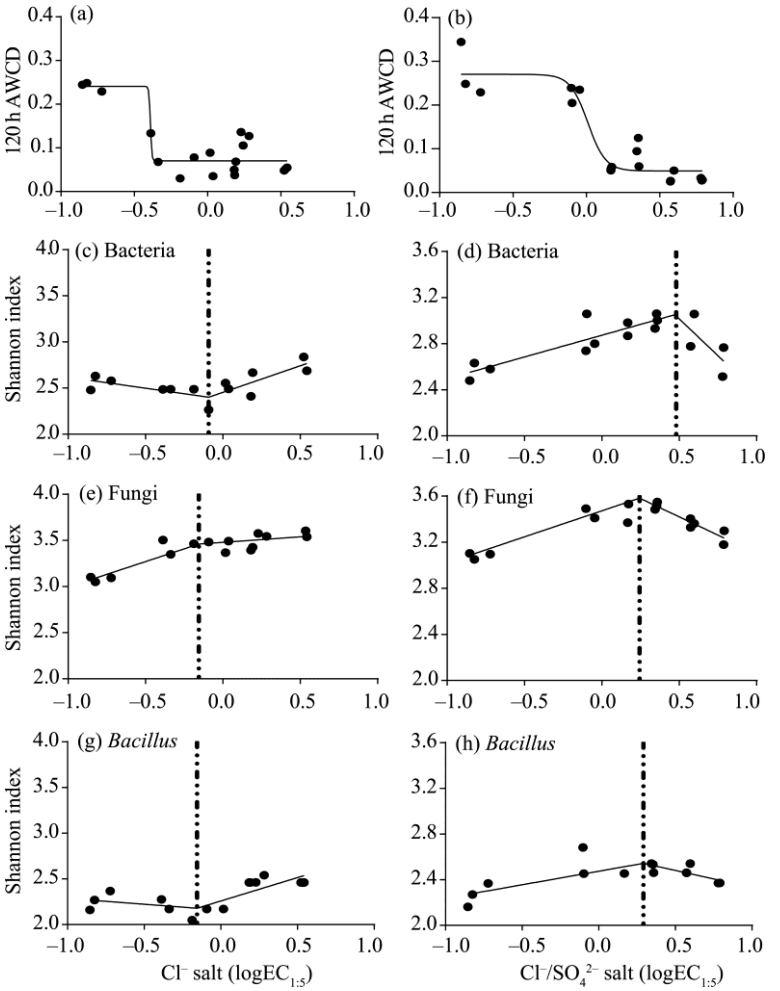

Fig. 5 Non-linear analyses of the average well color development (AWCD, 120 h) with Cl^- (a) and Cl^-/SO_4^{2-} (b) salts. Relationships between soil salt contents and the diversities of soil bacteria (c, d), fungi (e, f) and *Bacillus* (g, h). Diversities based on 16S-rDNA-PCR-DGGE banding profiles for soil total bacteria and *Bacillus*, and ITS-rDNA-PCR-DGGE for fungal banding profiles. Segmental dose-response logistic regression (EC with AWCD) and segmental linear regression (EC with diversity index) were used to determine breakpoints. Vertical dotted lines intersect the break-point of microbial diversity with respect to log of soil electrical conductivity ($logEC_{1:5}$).

Bacillus community to Cl^- treated soil (Table 4). Moreover, the community diversities of bacteria and *Bacillus* showed an opposite response to Cl^- salt (Figs. 5c and g), indicating that Cl^- salt exhibited strong stress to soil microbial community even at the lower application rate.

Table 4 Critical thresholds of community diversities of soil bacteria, fungi and *Bacillus* in responsive to Cl^- and Cl^-/SO_4^{2-} salts

Salt type	Community	Soil EC (dS/m)	95% CI	R^2	Added salt concentration (g/kg)	Segmental versus single regression[c]
Cl^- saline[a]	Biolog	0.45	0.33–0.61	0.83	0.89	P=0.0020
	Bacteria	0.81	–	0.62	2.64	P=0.0210
	Fungi	0.70	–	0.83	2.13	P=0.0325
	Bacillus	0.70	–	0.64	2.13	P=0.0146
Cl^-/SO_4^{2-} saline[b]	Biolog	1.26	1.21–1.87	0.89	5.25	P=0.0426
	Bacteria	3.03	–	0.70	17.10	P=0.0255
	Fungi	1.75	–	0.75	8.55	P=0.0007
	Bacillus	1.96	–	0.76	9.93	P=0.0397

Note: [a], chloride saline was a mixture of $CaCl_2$ and NaCl; [b], mixed saline was a mixture of $CaCl_2$ and Na_2SO_4; [c], comparison the fitting of the community diversities to soil EC induced by the Cl^- or Cl^-/SO_4^{2-} salt. Where P<0.05, segmental linear of AWCD and dose-response logistic regression for microbial structure diversity fitted the data significantly better than single linear regression alone. EC, electrical conductivity; 95% CI, 95% confidence interval; –, no data.

4 Discussion

In this study, the negative correlations between soil salt (Cl^- and mixed Cl^-/SO_4^{2-} salts) rates ($EC_{1:5}$) and the invertase activity (R^2=0.964 in Cl^- salt and R^2=0.674 in mixed Cl^-/SO_4^{2-} salt, n=18), MBC (R^2=0.600 in Cl^- salt and R^2=0.876 in mixed Cl^-/SO_4^{2-} salt; n=18) and SIR (R^2=0.760 in Cl^- salt and R^2=0.866 in mixed Cl^-/SO_4^{2-} salt; n=18) were obtained (Figs. 1 and 2). Our findings are in agreement with the report by Rietz and Haynes (2003) who discovered the enzymatic activities of catalase, invertase, and β-glucosidase decreased with soil EC increasing. Similarly, Ghollarata and Raiesi (2007) reported soil β-glucosidase, invertase, and urease activities were largely inhibited by soil salt. Following this, Yan and Marschner (2012) confirmed soil microbial activities including cumulative respiration and MBC were negatively affected by salinity. The most likely reasons why soil enzymatic activity decreased when exposed to salt ambient were as follows: (1) high salt concentration can result in a low availability of water due to decreasing soil osmotic and matric potential, as a consequence, resulting in the dehydration of soil microbes (Rath and Rousk, 2015). For example, Saviozzi et al. (2011) reported the decrease of soil enzyme activity was highly associated with the decrease of soil osmotic potential caused by salt; (2) soil salt denatured enzymatic proteins and accelerated soil enzymes proteolysis (Capriotti et al., 2014); and (3) soil aggregate was dispersed and soil structure was destroyed by salt ion, resulting in the deterioration of living inhabits for the soil microorganisms (Saviozzi et al., 2011).

The greater soil MBC and enzyme activity with mixed Cl^-/SO_4^{2-} salt than with Cl^- salt may be partly due to the relatively high toxic effect of Cl^- over mixed Cl^-/SO_4^{2-} ion on soil microbe, especially for soil bacteria (Rath et al., 2016). As such, Cl^- was more toxic than SO_4^{2-} on cellular protoplasm and the structure of bacterial extracellular polymeric substance. Our findings largely agreed with the report by Crisler et al. (2012) who discovered that Cl^--dominated environment was more harmful to soil microbe than SO_4^{2-}-dominated soil condition. Similarly, Batra and Manna (1997) found a stronger toxic impact of Cl^- instead of SO_4^{2-} on soil microbial activity.

In this study, SBR showed a faint response to either salt rates or salt type, while SIR (AWCD generated, 120 h AUC data) significantly decreased with increasing in salt rates, indicating a strong negative impact of salt rates on microbial activity and the functional diversity, and further confirming that it is SIR rather than SBR that more reliable on explaining the changes of soil

biological activity to saline-induced in EC. Our findings were in accordance with the reports by Mccarty et al. (2007) who noted that SIR was a sensitive indicator for soil microbial activity.

The diversities of soil bacteria, fungi, and *Bacillus* communities increased with mixed Cl^-/SO_4^{2-} salt increasing until reached a peak point and then sharply declined (Fig. 5); this may be partially owning to the fact that the growth of certain soil microbe species would be constrained in an intermediate level of salt stress, while other species may be stimulated; as the salt concentration exceeded 10 g/kg (10 Cl^-/SO_4^{2-} treatment, EC=2.25 dS/m; Fig. 2b), the stress of salt on soil microbial microorganism became too strong to tolerate or to survive, consequently, a large number of microorganisms would be dead (Fig. 3a). Thereby, the obvious reductions in microbial function and community diversities were observed (Figs. 2b, 3c, and 4), this was further supported by PERMANOVA analysis (P<0.05; Table 2). Our findings were lined up with the results by Mohamed and Martiny (2011), who sequenced 18S rRNA gene in estuarine salinity gradient sediment and showed that the composition of fungal community changed substantially, whereas fungal diversity altered only at the finest level, and the highest diversity was gained in the intermediate salt level.

In this study, we found that the response pattern of microbial community diversity in mixed Cl^-/SO_4^{2-} salt treatment showed a hump-shape exponential decay. Our findings agreed with the viewpoint by Giller et al. (2009) who found that the changes of soil microbial diversity did not linearly decrease with disturbance (e.g., metal, salt, etc.). Furthermore, the lowest value on maintaining fungal structural diversity was 1.75 dS/m in this study, which could be regarded as the boundary of soil salinization occurrence. Our data strongly supports the result of Crecchio et al. (2004) who stated that soil salinization occurred as soil EC exceeded 2 dS/m. As for Cl^- treated soil, the lowest threshold value of EC appeared at 0.70 dS/m for both soil fungi and *Bacillus* (Table 4), and it was coincident with the reduction in soil bacteria community diversity (0.81 dS/m) (Table 4; Figs. 5c, e and f), suggesting chloride salt alone led to a strong detrimental impacts on soil microbe, and resulting in a significant decrease in soil microbial diversity.

The dose-response inhibition effects of soil physiological metabolic profiles (Biolog) with either Cl^- (R^2=0.83) or mixed Cl^-/SO_4^{2-} (R^2=0.89) were detected, and the greater threshold value in maintaining soil physiological function was gained in mixed Cl^-/SO_4^{2-} (EC=1.26 dS/m) than in Cl^- (EC=0.45 dS/m) added soil, which further supported the above mentioned that Cl^- exhibited greater toxic effect than mixed Cl^-/SO_4^{2-} salt. In addition, the salt thresholds calculated from AWCD for Cl^- (EC=0.45 dS/m) and for mixed Cl^-/SO_4^{2-} (EC=1.26 dS/m) treatments were lower than the corresponding threshold values calculated from microbial communities diversity for Cl^- (0.70–0.81 dS/m) and for Cl^-/SO_4^{2-} (1.75–3.03 dS/m) (Table 3), respectively, indicating soil physiological function was more susceptible than soil microbial community diversity to the salt concentration.

5 Conclusions

Our findings suggest that the structures of soil bacterial, fungal, and *Bacillus* communities were obviously affected by both salt type and final soil EC, and soil salt exerted an adverse influence on soil enzyme activities, MBC and SIR (AWCD generated). The greater soil MBC and enzymes with mixed Cl^-/SO_4^{2-} salt than with Cl^- salt may partially attribute to the relatively low toxic effect of Cl^-/SO_4^{2-} ion over Cl^- on soil microbe. Furthermore, SIR was a more reliable indicator than SBR in explaining the changes of soil biological activity responsive to saline-induced EC. The threshold concentrations of salinity ($EC_{1:5}$) in maintaining soil microbial community diversity were 1.75 dS/m for mixed Cl^-/SO_4^{2-} and 0.7 dS/m Cl^- salts. Likewise, the threshold concentrations of salt in maintaining soil physiological function were 1.26 and 0.45 dS/m for mixed Cl^-/SO_4^{2-} and Cl^- salts, respectively, indicating soil physiological function was more susceptible than soil microbial community structural diversity to the salt level.

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